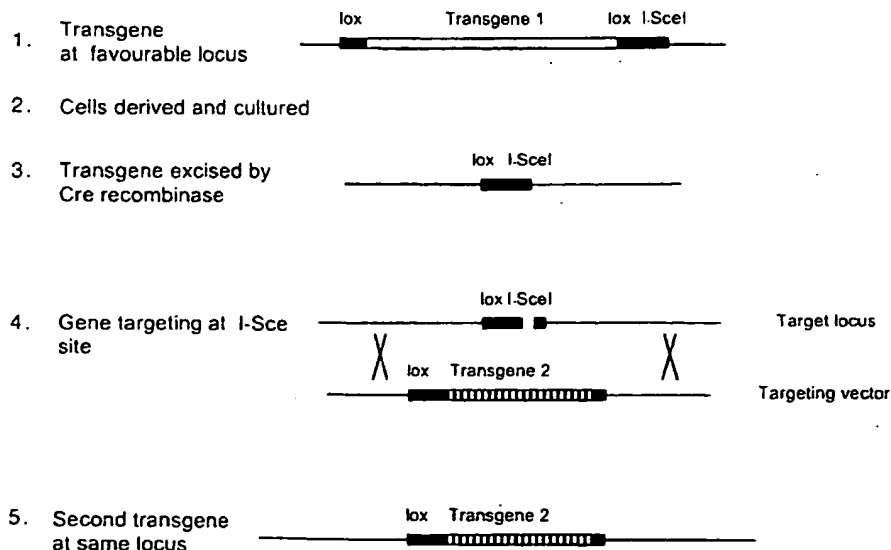




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(54) Title: PRODUCTION OF TRANSGENIC DONOR CELLS FOR NUCLEAR TRANSFER

TRANSGENE PLACEMENT AT A LOCUS MARKED BY
A RARE ENDONUCLEASE RECOGNITION SITE

(57) Abstract

The present invention relates to a process for producing a nuclear donor cell, a process for obtaining predictive information of the phenotype of a transgenic animal and a process for reconstituting an animal embryo, wherein all processes involve the screening of a cell and/or analysis of the phenotype of a cell, preferably analysis of transgene expression characteristic(s). The present invention also provides nuclear donor cells, reconstituted animal embryos and animals according to the process of the invention.

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PRODUCTION OF TRANSGENIC DONOR CELLS FOR NUCLEAR TRANSFER

The present invention relates to a process for producing a nuclear donor cell, a process for obtaining predictive information of the phenotype of a transgenic animal and a process for reconstituting an animal embryo, wherein all processes involve the screening of a cell and/or analysis of the phenotype of a cell, preferably analysis of transgene expression characteristic(s). The present invention also provides nuclear donor cells, reconstituted animal embryos and animals according to the process of the invention.

Genetic manipulation of domestic animals could bring numerous agricultural and medical benefits, including the introduction of desirable traits such as disease resistance or enhanced performance into food animals, the production of high volume biologically active human proteins for human therapy and donor tissues for human xenotransplantation. However, the production of transgenic domestic animals, such as sheep, pigs and cattle, by the current method of DNA microinjection into zygotes is time consuming, costly, limited in scope and the quality and quantity of transgene expression is uncertain.

Transgenic animals have been defined as "animals that have integrated foreign DNA into their germ-line as a consequence of experimental introduction of DNA" (Palmiter RD, and Brinster RL. Cell, 41, 343-345, 1985). Throughout the whole of this document, we use the term

"transgenic" in a broader sense to include animals in which any type of genetic modification (eg. gene deletion, mutation, substitution) has been carried out using, at some stage, genetic manipulation in vitro. A
5 description of the present invention is preceded by a review of currently available and prospective methods of producing transgenic animals.

Pronuclear microinjection: This method was first
10 described in 1980 (Gordon JW et al., *Proc. Natl. Acad. Sci. USA* 77: 7380-7384), and since then has been used extensively in many species. In short, naked DNA is microinjected into the pronucleus of explanted zygotes which are then transferred to foster mothers to complete
15 gestation. Transgene integration into the host genome is random and in mice typically occurs in 5-20% of offspring.

Pronuclear microinjection is by far the most widely used
20 method of gene transfer in livestock (Ebert KM and Schindler, *JES, Theriogenology* 39, 121-135, 1993).. However, the transgenic rate (<1-5% of offspring) is often significantly lower than mice and the costs considerably greater. It has been estimated that
25 approximately 1200 microinjected bovine zygotes are required to produce a single transgenic calf (Eyestone, W.H., *Reprod. Fertil. Dev.*, 6, 647-652, 1994). A straightforward application of the approach used in mice therefore requires approximately 300 super-ovulated cows
30 as zygote donors and 600 cows as recipients for microinjected embryos. Although methods have been

developed to reduce these numbers, there is still a considerable incentive to use animals more efficiently.

The use of donor animals for zygote production can be avoided by in vitro maturation and fertilization (IVM/IVF) of oocytes obtained from slaughterhouse material. This technique was first developed in cattle and has been used to produce transgenic calves (Krimpenfort, P., et al., *Bio-technology* 9, 844-847 1991; Hill, K.G., et al., *Theriogenology* 37, 222, 1992; Bowen, R.A., et al., *Biol. Reprod.* 50, 664-668, 1994). Although zygotes produced in vitro are less viable than those derived in vivo, this is offset by the greater number available. Further refinements of IVM/IVF and culture conditions can be expected to improve zygote quality in the future.

The number of recipient cattle can be minimised by identifying transgenic embryos either before transfer or during gestation. Polymerase Chain Reaction (PCR) analysis of embryo biopsies obtained prior to embryo transfer has been used to produce transgenic cattle (Haskell and Bowen, *Mol. Reprod. Dev.* 40, 1994). However, there is no method available which can reliably distinguish between integrated transgene and residual non-integrated DNA (Krisher, R.K., et al., *Theriogenology* 41, 229, 1994). Alternatively, transgenic fetuses can be identified in utero from samples obtained by amnio - or allantoctesis.

30

Improvements in the rate of transgene integration could

be achieved by optimal timing of zygote microinjection. Exogenous DNA is thought to integrate during DNA replication and thus microinjection should ideally be performed before or during early S-phase (Bishop, J.O. and Smith, P., *Mol. Biol. Med.* 6, 283-298, 1989). However, this is impractical in livestock species because of the difficulty of visualising zygote pronuclei. Attempts to synchronise bovine zygotes in S-phase before microinjection have had limited success (Gagnè, M., et al., *Mol. Reprod. Dev.* 41, 184-194, 1995).

Retrovirus mediated gene transfer: Infectious retroviral vectors derived from replication defective retroviruses can be used to transduce non-viral genes with high efficiency, into dividing cells in vivo or in vitro (Weiss, R., et al., *RNA tumor viruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1985).

20

Retrovirus mediated gene transfer has been used to produce transgenic animals of several species, e.g. mice (Soriano, P., et al., *Science* 234, 1409-1413, 1986); Stewart, C.L., et al., *EMBO J.* 6, 383-388, 1987) and chickens (Perry, M.M. and Sang, H.M. *Transgenic Res.* 2, 1125-133, 1993). Although this approach is potentially more efficient than pronuclear injection, its use in the derivation of transgenic large animals has been limited. Haskell and Bowen (Haskell, R.E., and Bowen, R.A., *Mol. Reprod. Dev.* 40, 386-390, 1995) injected cells producing replication defective virus into the perivitelline space

of bovine zygotes and achieved 7% transgenic fetuses at day 90.

Retroviruses do, however, suffer several disadvantages which severely limit their usefulness. The size of DNA transduced is limited and effectively restricts the use of retroviral vectors to cDNAs, which are generally poorly expressed as transgenes. Differential timing of retroviral integration and the possibility of several different independent integrations leads to the frequent production of mosaic animals, which can fail to transmit the transgene through the germ line. Insertion of retroviral long terminal repeats (LTRs) into the host genome can also cause activation of adjacent genes with possible deleterious effects. Perhaps the most serious problem with transgenic animals carrying retroviral vectors is the risk of producing replication competent virus by recombination. Uncertainty regarding this possibility excludes at present, the use of animals containing retroviral transgenes for most human applications.

Sperm-mediated DNA transfer: DNA uptake by spermatozoa of mice, pig, sheep, cattle, poultry, carp, blowfly and sea urchin has been demonstrated (Bachiller, D., et al., *Mol. Reprod. Dev.* 30, 194-200, 1991; Castro, F.O. et al., *Theriogenology* 34, 1099-1110, 1990; Gavora, J.S., et al., *Can. J. Anim. Sci.* 71, 287-291, 1991; Arezzo, F., *Cell Biol. Int. Rep.* 31, 391-404, 1989; Atkinson, P.W., et al., *Mol. Reprod. Dev.* 29, 1-5., 1991). DNA internalisation into the sperm head has also been shown

in mouse and cattle (Bachiller et al., op. cit. 1991; Atkinson, op. cit. 1991).

Lavitrano et al., (Cell 57, 717-723, 1989) have reported
5 the production of transgenic mice by artificial insemination using spermatozoa exposed to exogenous DNA.

This could provide a time and cost saving alternative for the production of transgenic animals and was initially received with great interest. However despite
10 efforts from many laboratories around the world, the results could not be repeated in mice (Brinster, R.L., et al., Cell 59, 239-241, 1989). A single transgenic calf with a slightly rearranged transgene (Schellander, K., et al., Animal Biotech 6, 41-50, 1995) and five
15 transgenic pigs also with rearranged transgenes have been reported (Sperandio, S., et al., Animal Biotech. 7, 59-77, 1996). It now seems that considerable development may be necessary before this is a realistic approach.

20

Embryonic stem cell mediated transgenesis: Mouse embryonic stem (ES) cells are pluripotent cells derived from the early embryo (Evans, M.J., and Kaufmann, M.H., Nature 292, 154-156, 1981; Martin, G.R., Proc. Natl.
25 Acad. Sci. USA 78, 7634-7638, 1981). ES cells can be grown and manipulated in vitro and then introduced into a pre-implantation stage host embryo by microinjection or aggregation, where they participate in the formation of a chimeric animal and can contribute to somatic and
30 germ cell lineages.

ES cells provided the first cell mediated method of transgenesis. DNA transfer into a cell intermediate, rather than directly into the embryo, has the advantage that genetic manipulation and analysis can be carried out *in vitro* before animals are produced. Thus, ES cells are a direct alternative to microinjection into zygotes for the production of random transgenics and have been used where DNA microinjection is problematic, eg. in the production of mice containing yeast artificial chromosomes (Pearson, B.E., and Choi, T.K., *Proc. Natl. Acad. Sci. USA* 90, 10758-62., 1993; Jakobovits, A., et al., *Nature* 362, 255-258, 1993). However, the most powerful application of ES cell mediated transgenesis exploits the ability of ES cells to support homologous recombination between exogenous DNA and chromosomal sequences at a relatively high frequency. Gene targeting by homologous recombination allows precise modifications to be made at predetermined sites in the genome and has been used extensively to effect a wide variety of genetic manipulations (reviewed by Ramirez-Solis, R. and Bradley, A., *Curr. Opin. Biol. Sci.* 5, 528-533, 1994 and by Brandon, E.P., et al., *Curr. Biol.* 5, 5, 625-634, 758-765, 873-881, 1995).

Although great efforts have been made to derive ES lines from other species, definitive ES cell lines remain elusive. There are reports of ES or ES-like cell lines derived from hamster (Doetschman, T.C., et al., *Dev. Biol.* 127, 224-227, 1988), mink (Sukoyan, M.A., et al., *Mol. Reprod. Dev.* 36, 148-158, 1993), sheep (Piedrahita, J.A., et al., *Theriogenology* 34, 879-901, 1990;

Tsuchiya, Y., et al., *Theriogenology* 41, 321, 1994), cattle (Sims, M.M. and First, N.L., 1993 *Theriogenology* 39, 313, 1993; Cherny, L.R., and Merei, J., *Theriogenology* 41, 175, 1994; Stice, S., et al., 5 *Theriogenology* 41, 304, 1994; Strelchenko, N. and Stice, S., *Theriogenology* 41, 301, 1994), pig (Piedrahita, J.A., et al., op. cit. 1990; Notorianni, E., et al., *J. Reprod. Fertil.* 43: 255-260., 1991; Talbot, N.C., et al., *In Vitro Cell. Dev. Biol.* 29, 543-554., 1993, 10 Gerfen, R.W. and Wheeler, M.B., *Anim. Biotechnol.* 6, 1-14 1995) and rhesus monkey (Thomson J.A. et al., *Proc. Natl. Acad. Sci.* 92, 7844-7848, 1995). In all of these cases, the limited definition of "cells which under the appropriate *in vitro* conditions, can differentiate along 15 at least three different lineages" was used to underpin the "ES cell" claim. The production of pig (Wheeler, M.B., *Reprod. Fertil. Devel.* 6, 1-6, 1994) and rat chimeras (Iannaccone, P.M. et al., *Dev. Biol.* 163, 288-292, 1994) have also been reported, although in neither 20 case has ES contribution to the germ line been demonstrated. An improved method of ES cell derivation by drug selection may facilitate future ES cell derivation from other species (McWhir, J. et al., *Nat. Genet.* 14, 223-226, 1996). However, at present, ES cell 25 technology remains restricted to the mouse.

If and when large animal embryonic stem cells do become available, their use in the production of chimeras in the same way as mouse ES cells will carry several 30 disadvantages. Farm animals have a long generation interval and the production and breeding of chimeras can

delay the analysis of transgene expression considerably. Chimera production can be avoided in mice by deriving animals entirely from ES cells e.g., by aggregation of ES cells with disadvantaged tetraploid embryos.

5 However, this method is exquisitely sensitive to the status of the cell used (Nagy, A., et al., *Proc. Natl. Acad. Sci. USA* 90, 8424-8428, 1993) and the usefulness of this method for large animal ES cells is unknown.

10 As discussed later, the lack of large animal ES cells may be circumvented by recent developments in nuclear technology.

Embryonic germ cells: Primordial germ cells (PGCs) are
15 the progenitors of the gametes. Matsui et al., (*Cell* 70, 841-847, 1992) and Resnick et al., (*Nature* 359, 550-551, 1992) first identified a combination of growth factors (Leukaemia inhibitory factor, steel factor and basic fibroblast growth factor) which promote long term
20 growth of mouse PGCs and their conversion to a cell type termed embryonic germ (EG) cells. EG cells closely resemble ES cells and are functionally equivalent for cell mediated transgenesis. EG cells can be manipulated *in vitro*, and then contribute to somatic and germ cell
25 lineages of chimeric animals (Stewart, I.C., *Dev. Bio.* 161, 626-628, 1994; Labosky, P., et al., *In: Germline development*: pp 157-178, Pub. Wiley, Chichester, 1994).

In contrast with the small number of cells available
30 from early embryos, PGCs can be isolated in relatively large numbers during their migration through the fetus

and their eventual location in the genital ridge. Thus, isolation of EG cells from farm animals might be a viable alternative to ES cell derivation. Several attempts have been made to isolate EG lines from cattle
5 (Cherny, L.R., and Merei, J., *op. cit.* 1994, Stokes, T.M., et al., *Theriogenology*, 41, 303, 1994) and rat (Mitani, T., et al., *Theriogenology*, 258, 1994). Blastocyst injection of cultured EG cells leads to production of mid-gestation chimeric bovine embryos
10 (Stokes, T.M., et al., *op. cit.* 1994), but no live-born animals have been reported. Again, recent developments in nuclear transfer may circumvent these problems.

Spermatogonial stem cells: There has been a recent
15 demonstration that transplanted spermatogonia can repopulate the testes of sterile, or sub-fertile recipients in mice (Brinster, R.L. and Zimmermann, J.W., *Proc. Natl. Acad. Sci. USA* 91, 11298-11302, 1994; Brinster, R.L. and Avarbock, M.R., *Proc. Natl. Acad.*
20 *Sci. USA* 91, 11303-11307 and rat (Avarbock, M.R. *Nat. Med.* 2, 693-696, 1996) and that rat spermatogonia transplanted into mice produce spermatozoa (Clouthier, D.E. *Nature* 381, 418-421, 1996). If a system can be developed which allows *in vitro* culture and genetic
25 manipulation of spermatogonia or spermatogonial precursors, this could provide a future means of transgenesis (Lovell-Badge, R., *Nat. Med.* 2, 638-639, 1996).

30 Nuclear transfer: The replacement of the nucleus of one cell with that of another was used most famously to

demonstrate that nuclei of differentiated and undifferentiated cells have equivalent developmental potential. In 1952, Briggs and King (*Proc. Natl. Acad. Sci. USA* 38, 455-463, 1952) transferred cell nuclei from
5 blastula stage embryos of the frog *Rana pipiens* to enucleated eggs, where they were able to direct normal development to feeding stage larvae. Later, nuclear transfer from larval intestinal cells of the toad *Xenopus laevis* led to the development of mature, fertile
10 individuals (Gurdon, J.B., and Uehlinger, V., *Nature* 210, 1240-1241, 1966).

Until recently, nuclear transfer in mammals has only been possible using nuclei from cells obtained directly
15 from early embryos, or subjected to very short periods in culture. Live animals have been produced by nuclear transfer from embryonic blastomeres into enucleated oocytes in pigs (Prather, R.S., et al., *Biol. Reprod.* 41, 414-418, 1989), cattle (Prather, R.S., et al., *Biol.*
20 *Reprod.* 41: 414-418, 1987; Bondioli, K.R. et al., *Theriogenology* 33, 165-174., 1990; Keefer, C.L. et al., *Biol. Reprod.* 50, 935-939, 1994; Sims, M. and First, N.L., *Proc. Natl. Acad. Sci. USA* 91, 6143-6147, 1994) and sheep (Smith, L.C., and Wilmut, I., *Biol. Reprod.*
25 40, 1027-1035, 1989). Mid-gestation bovine fetuses have been produced by nuclear transfer from cultured bovine embryo explants (Stice, S., et al., op. cit., 1994; Strelchenko, N. and Stice, op. cit. 1994). Nuclear transfer from late stage blastomeres into oocytes has
30 been unsuccessful in mice and live offspring have only been reported between blastomeres at similar

developmental stages, e.g. 4 or 8 cell blastomere nuclei into 2 cell stage cytoplasts (Robl, J.M., et al., *Biol. Reprod.* 34, 733-739, 1986; Tsunoda, Y., et al., *J. Reprod. Fert.* 82, 173-178, 1988).

5

Difficulties associated with mice may be related to the time at which the embryonic genome becomes transcriptionally active. Mouse embryos initiate transcription at the 2 cell stage, pigs at 4 cell, cows at 4-8 cell and sheep at the 8-16 cell stage (Prather, R.S., *J. Reprod. Fertil.* 48, 17-29, 1993). It is conceivable that the delay before genome activation allows the transplanted nucleus to be reprogrammed by its cytoplasmic environment.

15

The birth of sheep following nuclear transfer from embryonic cells cultured for long periods has been reported (Campbell, K.H.S., et al., *Nature* 380, 64-66, 1996). Cells originally derived from day 9/10 ovine embryonic disc were passaged up to 13 times in culture and then used as nuclear donors. The authors propose that the key to successful nuclear transfer is the induction of a quiescent state in the donor cell. In the quiescent state, termed G₀, cell cycle activity is absent, protein synthesis is reduced and changes occur to the chromatin (Whitfield, J.F., et al., In: *Control of cell proliferation* Vol 1, pp 331-335, eds Boynton, A.L. and Leffert, H.L. Academic Press, London, 1985). It has been proposed that these alterations may render the nucleus more susceptible to modification by the oocyte cytoplasm (Campbell, K.H.S., et al., op. cit.,

30

1996). Quiescence can be induced in nuclear donor cells by culture in the presence of reduced amounts of serum, termed serum starvation. These nuclear transfer techniques for differentiated cells have been described
5 for the first time in WO97/07669 and WO97/07668.

Whatever the method used to obtain transgenic animals, random integration of foreign DNA into the host chromosome is the norm. Integrated transgenes typically
10 occur as single copies, or tandem head to tail arrays at a single site apparently randomly located within the genome. Transgenes integrated at different sites can vary widely in expression level due to the influence of the local genetic environment (Wilson. C et al ., Ann.
15 Rev. Cell Biol. 6, 679-714, 1990). This position effect variation requires that many lines of transgenic large animals must be independently derived to obtain one with a suitable level of expression.

20 At present, practical considerations compel the use of transgenic mice to assay constructs which are designed for expression in large animals. However, the shortcomings of the mouse as a predictor of the expression level in a large animal are widely
25 recognised. For example, Carver et al., (Biotechnology, 11, 1263-1269, 1993) found a markedly different range of expression levels of human α -1 antitrypsin from the same construct in the milk of transgenic mice (0.3 - 21.3 mg/ml) and sheep (0.7 - 33 mg/ml). Velandar et al.,
30 (Proc. Natl. Acad. Sci. USA, 89, 12003-12007, 1992) obtained 1 mg/ml expression of human Protein C from

transgenic pigs using a construct which expressed poorly in transgenic mice. Similarly, the post translational processing of a foreign protein by the mouse mammary gland is a poor guide to that in large mammals (Drohan, 5 W.M., et al., *Transgenic Res.* 3, 355-364, 1994). Nevertheless in the absence of an alternative, the transgenic mouse is currently the most viable model system for construct development.

10 We have established that placement of a transgene at a chromosomal location which favours high expression can overcome the problem of the position effect and techniques of transgene placement by gene targeting in ES cells are established (Stacey, A.J., et al., *Mol.*
15 *Cell. Biol.*, 14, 1009-1016, 1994; Bronson, S.K. et al., *Natl. Acad. Sci. USA* 93, 9067-9072, 1996). Although the frequency of homologous recombination in somatic cells has not been examined as extensively as in ES cells, it is noteworthy that the first demonstration of successful
20 gene targeting in mammalian cells was not in ES cells but in a somatic cell line (Smithies, O., et al., *Nature* 1985, 317, 230-234). Since then, gene targeting in somatic cells has been demonstrated many times and various strategies have been devised for identifying and
25 enriching the products of rare homologous recombination events (Shesely, E.G., et al., *Proc. Natl. Acad. Sci. USA*, 88, 4294-4298, 1991. Williams, S.R., et al., *Proc. Natl. Acad. Sci. USA* 91, 11943-11947, 1994; Arbones, M.L. et al., *Nat. Genet.* 61, 90-97, 1994. Scheerer, J.B.
30 et al., *Mol. Cell. Biol.*, 14, 6663-6673, 1994; Hzhaki, J.E et al., *Nature Genetics*, 15, 258-265, 1997).

Homologous recombination is thought to occur as a consequence of double-strand break repair (Szostak, J.W., et al., *Cell* 33, 25-35, 1983). Gene targeting frequency has been shown to be increased by introducing a double strand break at the target locus by a site-specific endonuclease in human somatic cells and murine ES cells (Brenneman, M., et al., *Proc. Natl. Acad. Sci. USA*, 93, 3608-3612, 1996) (Smih, F. et al., 1995, *Nuc. Acid Res.* 23, 5012-5019). The availability of endonucleases, e.g. I-SceI, which cleave at rare (18bp) recognition sites (Jacquier, A., and Dujon, B., *Cell*, 41, 383-394, 1985) allow double-strand breaks to be created at unique target loci in the host genome (Rouet, P., et al., *Mol. Cell. Biol.* 14, 8096-8106, 1994). Homologous recombination between exogenous and chromosomal DNA at a I-Sce site has been demonstrated in mouse fibroblasts (Choulika, et al., *Mol. Cell. Biol.* 15, 1968-1973, 1995).

The ability to maintain cells for long periods in vitro before nuclear transfer opens a new route for transgenesis. Donor cells can be genetically manipulated and optionally screened in vitro for the required genotype (ie. stable integrant). The chosen genotype can be transferred to whole animals by nuclear transfer. This approach is particularly suitable for large animals because, in contrast with ES cells, the animals are derived completely from the donor cell, not chimeras. However, a problem associated with selecting donor cells on the basis of genotype is that genotype

does not necessarily give information about the phenotype of the cell or of an animal derived from that cell. Thus, selection of donor cells on the basis of genotype is not an effective route for the production of transgenic animals with a desired phenotypic trait. It has not previously been thought possible to analyse the phenotype of nuclei which are to be used in the production of transgenic animals.

- 10 The present invention describes how it is possible to obtain such phenotypic information of nuclei to be used in the production of transgenic animal and thus describes a significant improvement in the production of transgenic animals with a desired phenotypic trait.
- 15 Furthermore, it is shown that nuclear transfer from cultured cells is not limited to a particular cell type such as that described by Campbell et al., (op. cit., 1996). As is demonstrated, cells of various tissues can be used successfully. This provides the major advantage
- 20 that a variety of donor cells can be chosen to provide predictive information about transgene expression in the whole animal.

We describe a method of cell mediated transgenesis in which a transgene is analysed in cultured cells which model the tissue of interest. This approach is applicable to a variety of tissues, for example fibroblasts and endothelial cells.

- 30 In the basic conception, a transgene construct is introduced into cultured cells, cell clones are derived

and individually analysed for transgene expression. Clones selected for desirable transgene expression are then used as nuclear donors to produce transgenic animals.

5

Accordingly, the present invention provides, as a first aspect, a process for producing a nuclear donor cell, the process comprising, transfecting a cell with a transgene and screening the cell for a desired
10 phenotype. As described previously in this text, the term "transgene" includes any type of genetic modification (deletion, mutation, substitution) and includes integration of foreign DNA and of endogenous DNA. In this text, the terms transfection and
15 "transforming" mean the introduction of nucleic acid (usually DNA) into a cell by any means, including micro injection and other techniques described in the introductory section. The process for producing the nuclear donor cell may further include assessing the
20 cell for suitability for nuclear transfer, such as testing the cell for its ability to survive serum starvation and/or establishing a normal chromosomal complement. The donor cell may be, but does not have to be, in culture.

25

The screening of the cell for a desired phenotype is preferably carried out *in vitro* but may also be carried out *in vivo*. The screening for the desired phenotype is preferably to determine, or to screen for transgene
30 expression characteristics. This is a specific part or area of a cell's phenotype. It most accurately reflects

information (qualitative and quantitative) about the transgene. The determination or screening of/for transgene expression characteristics is preferably by analysis of transgene RNA expression or by analysis of
5 transgene protein expression. Such analysis can give qualitative and quantitative information concerning the transgene. These two types of analysis currently provide the optimal basis for determining the phenotype of a transgenic animal produced from the nucleus of such
10 a cell.

The cell for transfection and screening may be any cell, including adult somatic cells, embryonic somatic cells or foetal cells, which can act as competent nuclear
15 donor cells. The invention relates to at least a partially differentiated cell (e.g. post-embryonic stage) and fully differentiated cells.

Advantageously, the transfected cell naturally models a
20 tissue of interest or is genetically engineered to model a tissue of interest. The cells may require addition of a suitable stimulus in order to model a tissue of interest. The ability to respond to a particular stimulus may be required. This can include a portion of
25 the transgene construct. The present invention includes the modification of cells so that they can provide a predictive model. For example, any modification can be made where genes are added to confer particular tissue specific properties. Specific embodiments include the
30 expression using the prolactin signalling pathway (prolactin receptor, STAT 5, JAK2 and optional other

factors) in fibroblasts enabling otherwise unresponsive fibroblasts to express milk genes in response to prolactin, and the expression of steroid hormone receptors, which, in the presence of a steroid hormone, e.g. estradiol, can activate transcription of genes with the appropriate response element. Accordingly, genetic modification may involve the introduction of those components of a signal transduction pathway required for tissue specific gene expression. Signal transduction pathways for tissue specific gene expression in several tissues have been elucidated in detail, e.g. prolactin induced gene expression in mammary epithelium, interleukin 2 induced gene expression in lymphocytes and iron induced gene expression in a wide variety of cell types. Alternatively, genetic modification may be achieved by the introduction of key regulatory factors which induce tissue specific gene expression. Examples of this are the muscle transcriptional activators myoD (Davis, R.L. et al, Cell 51, 987-1000, 1987) and myf 5 (Brown T. et al. EMBO J. 8, 701-709, 1989) which induce muscle specific gene expression when transfected into fibroblasts.

The process for producing the nuclear donor cell may further include the step of preparing the cell for nuclear transfer, e.g. by inducing the nuclear donor cells into a quiescent state, or synchronising the cell component activities, or any other method.

The process may also include a step of transgene placement at a favourable site. This process is

summerized here with a full description given under the advantages of the invention following a discussion of the particular aspects. For example, the transgene for transfecting the cell may be flanked by a recognition site for a site specific recombinase and a recognition site for a rare cutting endonuclease. An example of the site specific recombinase is the bacteriophage P1 Cre recombinase LoxP site and an example of a rare cutting endonuclease site is the site for I-Sce I. Such a transgene construct enables replacement of the first transgene with a second transgene at a specific target locus.

According to a second aspect of the invention there is provided a nuclear donor cell produced by a process of the first aspect of the invention. Such a nuclear donor cell can be used for nuclear transfer, for example as previously described by Campbell, K.H.S. et al., op. cit. 1996 and in WO97/07669 the content of which is fully incorporated herein by reference. All preferred features of the first aspect also apply to the second aspect.

According to a third aspect of the invention there is provided a process for obtaining predictive information of a phenotype of a transgenic animal (at any stage in its development), the process comprising transfecting a cell with a transgene and screening the cell to obtain phenotypic information. The process according to the third aspect of the invention may further include preferred features according to the first or second

aspect of the invention.

According to the fourth aspect of the invention there is provided a process for reconstituting an animal embryo, the process comprising, transfecting a cell with a transgene, analysing the phenotype of the cell, inducing the cell into a state suitable for nuclear transfer, such as a quiescent state and transferring the nucleus of the cell into a suitable recipient cell. A basic process for reconstituting an animal embryo is described by Campbell, K.H.S. et al., op. cit. 1996 and in WO97/07669. The recipient host cell is preferably an enucleated metaphase II oocyte, an enucleated unactivated oocyte or an enucleated preactivated oocyte. Enucleation may be achieved physically, by actual removal of the nucleus, pro-nuclei or metaphase plate, or functionally, such as by the application of ultraviolet radiation or another enucleating influence.

Suitable recipient enucleated oocytes are: Metaphase Arrested G1/G0 Accepting Cytoplasm; G0/G1 Activation and Transfer (Campbell et al., Biol. Reprod. 49, 933-942 (1993); Universal Recipient (Campbell et al. Biol. Reprod. 649, 933-942 (1993), Biol. Reprod. 50, 1385-1393 (1994).

25

On identification and selection of a suitable donor and a suitable recipient, the nucleus of the donor requires transfer to the recipient cell. This can be established by fusion (e.g. exposure of cells to fusion-promoting chemicals, such as polyethylene glycol, the use of inactivated virus (such as Sendai virus) or by the use

30

of electrical stimulation) or by other techniques such as microinjection (Ritchie and Campbell, J. Reproduction and Fertility, Abstract Series No. 15, p60).

- 5 Before or after nuclear transfer it may be necessary to struvilate the recipient cell into development. This may be done by pathenogenetic activation.

Preferred analysis of the phenotype of the cell is by
10 analysis of transgene RNA expression or analysis of transgene protein expression. Such analysis can give qualitative and quantitative information concerning the transgene. All preferred features of the first to third aspects also apply to the fourth.

15

Any cell can be used according to the first to fourth aspects of the invention. Preferably, the cell is a mammary epithelial cell (foetal or other), a fibroblast cell, an endothelial cell or a sub-endothelial cell.

20

In principle, all aspects of the invention are applicable to all animal cells and animals including birds, such as domestic fowl, amphibian species and fish species. In practice however, the present invention is
25 most applicable to non-human animals, especially non-human mammals, and placental mammals.

It is with ungulates such as cattle, sheep, goats, water buffalo camels and pigs that the invention is likely to
30 be most useful. The invention is also likely to be applicable to other economically important animal

species such as, for example, horses, llamas or rodents e.g. rats, mice or rabbits.

Preferably, the process according to the fourth aspect
5 of the invention reconstitutes an ungulate species embryo, more preferably a non-human, such as a cow, bull, pig, goat, sheep, camel or water buffalo animal embryo.

10 A suitable recipient cell for the fourth aspect of the invention, is an oocyte. The oocyte is advantageously enucleate.

Preferably, the transfected cell, according to the
15 fourth aspect of the invention, models a tissue of interest, either naturally, or by genetic modification, as described above according to the first aspect of the invention.

20 Advantageously, the transgene of the cell can be used to place an alternative transgene at a favourable site. For example, the transgene may be flanked by a recognition site for a site specific recombinase and a recognition site for a rare cutting endonuclease, as
25 described according to the first aspect of the invention.

The fifth aspect of the invention provides a reconstituted animal embryo produced by a process
30 according to the fourth aspect of the invention. The reconstituted animal embryo may be developed to term to

produce a transgenic animal. Such an animal is a sixth aspect of the present invention. All preferred features of aspects one to four also apply to aspects five and six.

5

The seventh aspect of the invention provides a process for producing an animal, the process comprising, reconstituting an animal embryo according to the process of the fourth aspect of the invention, causing such an animal to develop to term from the embryo and optionally breeding from the animal. All progeny/offspring from any animal of the present invention is also covered and is part of the present invention. Accordingly any animal derived from any animal of the invention is also part of the invention. The step of causing the animal to develop to term may be done directly or indirectly. In direct development, the embryo is allowed to develop to term without further intervention beyond that necessary to allow development to term to take place. Indirect development includes further manipulation of the embryo before full development takes place. One example of such a manipulation includes splitting the embryo and the cells clonally expanded for the purposes of improving yield.

25

All preferred features of aspects one to six also apply to the seventh (in particular the animals which may be produced).

30 An eighth aspect of invention provides an animal produced by the process of the seventh aspect of the

invention.

All preferred features of aspects one to seven, also apply to the eighth aspect.

5

A ninth aspect of the invention provides a process for targeting a transgene to a location of interest. The process may comprise: selecting a nuclear donor cell produced by the second aspect of the invention, specifically, wherein the transgene is flanked by a recognition site for a site specific recombinase and a recognition site for a rare cutting endonuclease; or selecting a reconstituted animal embryo which has been produced by the fourth aspect of the invention which includes a transgene which is flanked by the same loci; or an animal developed from such a reconstituted animal embryo, and using a gene targeting vector, preferably isogenic to the host cell, to place a target transgene at the target locus. Other processes for targeting a transgene to a location of interest may be used.

All preferred features of aspects one to eight also apply to the ninth aspect.

25

A tenth aspect of the invention provides a nuclear donor cell, a reconstituted embryo or an animal produced by the ninth aspect of the invention.

30 All preferred features of aspects one to nine also apply to the tenth.

The present invention provides several advantages over the production of transgenic animals by DNA microinjection:

5

1. Predictive information on transgene expression can be gained from cells of the species of interest, rather than from another species, typically mouse.
- 10 2. Analysis of transgenes in cultured cells is faster than in whole animals, thus speeding the process of transgene design.
- 15 3. A greater number of independent transgene integration sites can be analysed in cultured cells than in whole animals, increasing the chance of finding a favourable site.
- 20 4. This information can be obtained before any transgenic animals are made, thereby contributing to a much less expensive and labour intensive process.
- 25 5. Transgenic animals produced from a cell clone are genetically identical, thus phenotypic variation can be minimized.
- 30 6. Large numbers of identical animals can, in principle be generated from a single cell clone leading to the possibility of raising a production herd/flock in one generation. Currently,

production flocks are generated by breeding from a single founder animal, a process which takes several years.

- 5 7. These factors combine to ensure that the number of experimental animals used is kept to a minimum.

The feasibility of cell mediated transgenesis for any particular application depends largely on the
10 characteristics of the primary cells available from the tissue of interest. Ideally, cells should fulfil three requirements.

1. They should be free of any genetic aberration which
15 would prevent successful nuclear transfer.
2. They should be capable of growth in culture for sufficient time to undergo genetic manipulation and remain free of genetic aberration.
- 20 3. They should model the parameters of interest, or be capable of modification to provide a model, sufficient to provide predictive information concerning the construct and the transgenic animal.

25

There are numerous examples of cells in culture used to model aspects of the intact tissue including gene expression. For example keratinocytes (Watt, F.M. *FASEB J.*, 5, 287-294, 1991, Dubertret, L. *Skin Pharmacol.* 3,
30 144-148, 1990), hepatocytes (Ulrich, R.G. et al., *Toxicol. Letters*, 82-83, 107-115, 1995) and myoblasts

(Daubas, P., et al., *Nuc. acids res.* 16, 1251-1271, 1988). Therefore a wide variety of cell types from many species are available as potential predictors of transgene expression and nuclear donors. A preferred
5 embodiment of the invention is the use of cultured cells to predict lactation specific expression in the mammary gland of transgenic animals. In this respect it is particularly interesting that *in vitro* models of mammary epithelium have been established in mouse (Barcellos-
10 Hoff, M.H., et al., *Development* 105, 223-235 1989; Seely, K.A. and Aggeler, J., *J. Cell. Physiol.* 146, 117-130, 1991), sheep (Finch, L.M.B. et al., *Biochemical Society Transactions* 24, 3695, 1996) and goat (Wilde, C.J. et al., *Biochemical Journal* 305, 51-58).

15

The type of genetic manipulation that can be carried out will depend upon the characteristics of the cells. While addition of a randomly integrated transgene is possible in any cell type, gene targeting requires a
20 frequency of homologous recombination sufficient to identify targeted events against a background of random integrants, as discussed previously. The present invention also includes the situation where transgenic animals can be made by conventional pro-nuclear
25 injection and nuclear-transfer cell lines can be derived from animals shown to express a transgene optimally in the tissue of interest (e.g. mammary gland). If the location of the transgene (by conventional techniques or techniques according to the present invention) is judged
30 to be favourable for expression, there are several possible schemes by which other transgenes can be placed

at the same site.

A site which supports favourable expression is identified using a transgene construct linked to a counter-selectable marker, e.g. the Herpes simplex tk gene, loss of which can be selected by the drug ganciclovir or the HPRT (Hypoxanthine phosphoribosyl transferase) gene, loss of which can be selected by the drug 6-thioguanine in cells lacking endogenous HPRT activity, or the Aequoria Victoria green fluorescent protein gene (Chalfie, M. et al., 1994, Science 263, 802-805) loss of which can be detected visually. DNA regions flanking the integrated transgene are cloned and incorporated into a replacement gene targeting vector.

15

Somatic cells containing a transgene plus marker construct at the desired site are derived either from the original clone, or re-derived from a transgenic animal produced from that clone. Re-derivation of early passage cultures may be preferable as this minimises the time in culture and any consequent deleterious effect on nuclear transfer.

Homologous recombination between the replacement targeting vector and the target locus can then be used to place a second transgene at the same site. Targeted events in which the counter-selectable marker is lost are either drug selected (Stacey et al., Mol. Cell. Biol. 14, 1009-1016, 1994), or identified visually as lacking GFP expression.

30

As mentioned previously, the efficiency of gene targeting in somatic cells has not been thoroughly investigated. Figure 1 shows how the efficiency of gene targeting can be enhanced by placement of a rare cutting
5 endonuclease recognition site at the target locus.

1. A site which supports favourable expression is identified using a transgene construct which is flanked by recognition sites for a site specific recombinase
10 (e.g. bacteriophage P1 *Lox P* sites recognised by Cre recombinase Sauer, B., *Methods Enzymol.* 225, 890-900, 1993), or yeast *FRT* sites recognised by *FLP* recombinase (Flering et al., *Proc. Natl. Acad. Sci. USA.*, 90, 8469-8473, 1993)). These are flanked on one side by a
15 recognition site for a rare cutting endonuclease, e.g. *I-SceI*. In the following example *LoxP* sites and *I-SceI* site have been used. A counter-selectable marker could be included within the region flanked by the recombinase recognition sites, but, given the high efficiency of
20 recombinase mediated excision, may not be necessary. The effect of an adjacent marker gene must also be taken into account when assessing transgene expression level.

2. Somatic cells containing a transgene construct at
25 the desired site are derived either from the original clone, or re-derived from a transgenic animal produced from that clone.

3. The first transgene is excised by the action of Cre
30 recombinase, which can either be introduced into cells as protein, or expressed from a transfected DNA

construct.

4. A gene targeting vector (possibly including a selectable marker e.g. neo) is used to place a second
5 transgene at the target locus. I-SceI endonuclease, introduced into cells with the targeting vector, is used to enhance the efficiency of targeting by double strand cleavage at the I-Sce site at the target locus. Illustrated in Figure 1 is a replacement type vector,
10 alternatively an insertion type vector could be used.

5. Cells confirmed as containing a transgene at the target locus are used to generate a second transgenic animal.

15

The present invention contemplates the production of transgenic animals which carry antibiotic resistance. Such transgenic animals may be undesirable because of the risk that drug resistance may be transferred to
20 pathogenic prokaryotic organisms. Several possibilities exist to reduce this risk.

1. The selectable marker is removed from cells in vitro before nuclear transfer. This can be
25 achieved by excision at flanking site specific recombinase recognition sites, e.g. the bacteriophage P1 Cre *LoxP* system (Sauer, B., *Methods. Enzymol.* 225, 890-900, 1993). Removal of a selectable marker by this method would, of
30 course, use a site specific recombinase system different from any involved in transgene

replacement.

2. DNA transfer is carried out by a method which does not require drug selection, e.g. DNA microinjected
5 into cells, individual clones derived and assayed for the presence of the transgene.
3. The selectable marker used can be modified such that it is nonfunctional in prokaryotic cells.
10 For, example the neo expression cassette used in the PGK neo construct already lacks 5' elements required for bacterial expression. This gene could be modified further by the introduction of a eukaryotic intron to render it non-functional in
15 prokaryotes.
4. Selectable markers can be used which are non-functional in prokaryotes, e.g. histidinol selection for the his gene (Hartman, S., et al.,
20 Proc. Natl. Acad. Sci USA, 77, 3567-3570, 1980).

Alternatively, the single recombinase recognition site left after step 3 in the scheme shown in Figure 1 can be used to target integration of a second transgene to that
25 site by site-specific recombination. In this case a second transgene construct containing a single recombinase recognition site is introduced into the cells as a circular episome. Recombinase mediated recombination between these two sites results in
30 integration. The efficiency of this reaction relative to the reverse excision reaction has not been clearly

established, but integration of a transgene into cultured cells by this method has been reported (Rucker, E.B. et al., *Theriogenology* 47, 228, 1997).

5 The present invention will now be described with reference to the following, non-limiting drawings:

Figure 1

Schematic representation of transgene placement by gene
10 targeting at a locus marked by a rare endonuclease recognition site.

Figure 2

Microsatellite analysis of nuclear transfer cells and
15 lambs.

Each of the 3 panels shows polymerase chain reaction amplification products from genomic DNA samples. The identity of each microsatellite sequence is indicated to
20 the right.

Lanes, A-G embryo transfer recipient ewes; lane H, SEC1 cells; lane I-L, lambs born from SEC1 nuclear transfer; lane M, BLWF1 cells; lanes N, O, lambs born from BLWF1
25 nuclear transfer.

PCR products were separated by electrophoresis on 4% metaphor agarose (FMC) and visualised by ethidium bromide fluorescence. PCR primers and reaction
30 conditions used were as described by Buchanan, F.C. et al., (*Mammalian genome* 4, 258-264; 1993).

Figure 3

Sex determination of nuclear transfer cells and lambs.

5 Each lane shows amplification products from samples of genomic DNA using a combination of two primer pairs: one designed to amplify a portion of the SRY gene present on the Y chromosome, and one designed to amplify a portion of the ZFX gene present on the X chromosome. Thus, the
10 presence of two amplification products indicates a male, one amplification product indicates a female.

Lanes A, B, male and female control DNA; lanes C-I, embryo transfer recipients; lane J, SEC1 cells; lane K-N
15 lambs born from SEC1 nuclear transfer; lane O, BLWF1 cells; lanes P, Q, lambs born from BLWF1 nuclear transfer. Individual identification numbers of each sheep are shown over each lane.

20 PCR products were separated by electrophoresis on 2% agarose and visualised by ethidium bromide fluorescence. Primer sequences and amplification conditions were as described by Griffiths, R. et al., (*Molecular Ecology*, 2, 405-406, 1993) and Horvat, S. et al., (*Transgenic*
25 *Research*, 2, 134-140, 1993).

Figure 4

Construction of PGKSV40Tts

30 The present invention will now be described by the following non-limiting examples. In the following

examples we describe cell mediated transgenesis using three cell types: adult ovine mammary epithelial, fetal ovine fibroblast and embryo derived cells, and we describe methods by which cells can be modified to
5 improve their suitability for cell mediated transgenesis.

Example 1. Live sheep produced by nuclear transfer from
10 fetal fibroblast cells

This example demonstrates that live sheep can be produced by nuclear transfer from fetal fibroblast cells which have been maintained in vitro.

15 Fibroblast cells, termed BLWF1, were prepared by mechanical and enzymic disaggregation of a Black Welsh ovine fetus at 26 days of gestation and cells grown on tissue culture plastic. After 4 passages the modal chromosome number was assessed as 54.

20

BLWF1 cells at passage 4-6 were prepared for nuclear transfer by serum starvation and nuclear transfer carried out as described by Campbell et al., (op. cit., 1996) results are shown in Table 1:

25

Table 1 Nuclear transfer from fetal fibroblast cells

Cell Type BLWF1

5	Number of reconstructed embryos	172
	Number of morulae + blastocysts	47
	Number of embryos transferred	40
	Number of pregnancies	5
	Number of live lambs	3

10

The three lambs born were all Black Welsh in appearance. Although one died within minutes of birth, the other two were normal and healthy. DNA analysis of the two living lambs showed them to be male (Figure 3) and identical to

15 BLWF1 cells by microsatellite analysis using 4 primer pairs.(Figure 2).

Example 2. Transgenic sheep produced by nuclear

20 transfer from fetal cells

This example demonstrates the production of transgenic sheep by cell mediated transgenesis using fetal fibroblasts.

25 Seven strains of primary ovine cells, termed PDFF (Poll Dorset Fetal Fibroblast) 1-7, were derived by disaggregation of fetuses at day 35 of gestation and cultured as described by Campbell et al., (op. cit., 1996) Stocks of early passage PDFF cells were

30 cryopreserved to provide cells when required.

Ovine fetal fibroblast cells are particularly suitable for cell mediated transgenesis and nuclear transfer because they are readily available in large numbers, grow rapidly and sustainably in culture for 12 weeks, are readily transfectable, maintain euploidy after long term culture (Table 2) and survive serum starvation.

Table 2 Chromosome counts of PDFF2 cells after extended passage

Cells	Passage	%54	%54+53
PDFF non transfected	p7	71.4	70
PDFF non transfected	p12	55.6	
PDFF non transfected	p19	65.5	

PDFF2 cells were stably transfected with a milk specific transgene pMIX1 and a selectable marker, clones were derived, analysed and suitable clones used as nuclear donors to produce transgenic sheep. The procedures used were as follows:

20

At first passage, 2×10^5 PDFF2 cells were plated into a 3.5cm diameter well and stably transfected using cationic charged liposomes with a milk specific transgene pMIX1. pMIX1 consists of the human Factor IX gene (31.37Kb), from 12bp upstream of the translational start signal to 12bp downstream of the translational stop signal, flanked at the 5' end by a 4.2Kb BLG promoter region and at the 3' end by a 2.2Kb BLG region containing 3' untranslated sequence polyadenylation signal and non-transcribed region. This is contained within the bacterial pUC18 vector. pMIX was

cotransfected with a selectable marker construct PGKneo in which the neo gene, conferring resistance to the drug G418 (Colbère-Garapin et al., *J. Mol. Biol.* 150, 1-14, 1981), is under the control of the human phosphoglycerate kinase promoter.

Because the effect of drug selection and growth as single cell clones on the ability of cells to support nuclear transfer were unknown, cells were treated in two ways. One group was grown at high density under G418 selection, then cryopreserved as a pool for nuclear transfer. The other group was plated at low density under G418 selection and cloned transfectants grown from isolated colonies. 48hrs after transfection cells were split 1:10 and G418 added to 0.6mg/ml. Transfected PDFF2 cells reached subconfluence after 6 days selection. At third passage one portion of the cells was split 1:10, subjected to a further 5 days selection and cryopreserved as an uncloned population. Other portions were split 1:1000 and 1:5000 and subjected to G418 selection for a further 7 days. 24 individual colonies were isolated, a sample cryopreserved at passage 5 and each clone grown further for Southern hybridization analysis. 21 clones were analysed, of which ten clones were found to contain pMIX1. The chromosome numbers of the four most rapidly growing clones are shown in Table 3.

Table 3 Analysis of PDFF cell chromosome number

The figures give the percentage of metaphase spreads which showed a chromosome count of 54 or 53 from four
 5 stably transfected clones

Cells	Passage	%54	%54+53
PDFF2 pMIX clone 12	p6	88.5	92.3
PDFF2 pMIX clone 13	p6	39.4	75.8
10 PDFF2 pMIX clone 31	p6	59	70
PDFF2 pMIX clone 38	p6	31.25	50

Nuclear transfer was carried out using PDFF2 transfected clones 12 and 13 as nuclear donors. Non-transfected PDFF
 15 (PDFF5) cells and pools of PDFF2 transfectants were also used to provide controls for the differences in cell strain the effects of transfection and growth from a single cell on nuclear transfer. Cells at passage 3-6 were prepared for nuclear transfer by serum starvation
 20 for 5 days and nuclear transfer performed as described by Campbell et al ., (op. cit. 1996). Results are shown in Table 4.

Of the six liveborn lambs produced from transfected
 25 cells, both from clone PDFF2-12 and that from clone PDFF2-13 contained both pMIX1 and PGKneo transgenes, the three lambs from the pool of transfected PDFF2 cells contained the PGKneo selectable marker only.

Table 4 Results of Nuclear Transfer

	PDFF5 non- transfected	PDFF2 pool	PDFF2- 12	PDFF2- 13
Reconstructed embryos	82	224	89	112
No. developed to morulae or blastocysts	5 (6.1%)	22 (9.8%)	19 (21.4%)	23 (20.5%)
Embryos transferred	5	22	19	21
Recipients	2	9	7	6
Pregnancies at day 60	2	4	4	1
Fetuses at d60 (% of embs. transf.)	3 (60%)	4 (18.2%)	6 (31.6%)	1 (4.8%)
Liveborn lambs* (% of embs. transf.)	1 (20%)	3 (13.6%)	2 (10.5%)	1 (4.8%)
Nuclear transfer efficiency (% live lambs from reconstructed embryos)	1.22%	1.34%	2.25%	0.89%

* Liveborn lambs were defined as those with a heart beat and able
5 to breath unassisted at birth.

This confirms that primary somatic cells can undergo
genetic manipulation, transfected cell clones can be
isolated and analysed in vitro and that viable animals
10 can be produced from those cell clones by nuclear
transfer.

Example 3. Primary fetal cells may be modified to allow analysis of transgene expression

This example demonstrates how cultured cells can be modified to provide predictive information on transgene
5 expression and how that information can inform the choice of transfected cell clones used for nuclear transfer.

Although fetal fibroblast cells are suitable for cell mediated transgenesis, they are unlikely, without
10 modification, to express genes designed for expression in mammary epithelium. However, such cells can be genetically modified to support milk gene expression. This has been previously demonstrated with other cell types which do not normally express milk genes.

15 Chinese hamster ovary cells can be stably transfected with the rabbit prolactin receptor and support expression of the mammary specific rat β -casein and ovine β -lactoglobulin promoters (Lesueur, L. et al., *Proc. Natl. Acad. Sci. USA* 88,824-828, 1991; Demmer, J.
20 et al., *Mol. Cell. Endocrinol.* 107, 113-121, 1995), similarly, NIH3T3 mouse fibroblasts can be transfected with the human prolactin receptor and support expression of the rat β -casein promoter (Das, R. and Vonderhaar, B.K., *Mol. Endocrinol.* 9, 1750-1759, 1995).

25

Here we describe two approaches by which fetal fibroblast cells can be modified to support the expression of milk genes.

A. Introduction of individual components of the prolactin signal transduction pathway into fibroblasts. Components of the prolactin signal transduction pathway which have been identified are the long form of the prolactin receptor (PRLR), Signal Transducer and Activator of Transcription 5 (STAT5) and Janus kinase 2 (JAK2) (Watson, C.J. and Burdon, T.G. *Rev. Reprod.* 1 1-5, 1996, Groner, B. and Gouilleux, F. *Curr. Opinions Gen. and Dev.* 5, 587-594). Although several of these factors are expressed individually in other cell types, induction of milk gene expression via this pathway is a specific characteristic of mammary epithelium.

Table 5 shows mammary specific reporter gene expression from fetal fibroblasts modified by the addition of cloned components of the prolactin signal transduction pathway in two repeat experiments. PDF2 cells were stably cotransfected with constructs designed to express the prolactin receptor (long form of the rabbit prolactin receptor cDNA driven by SV40 promoter early regions) and mouse STAT5a (STAT5a cDNA driven by a CMV promoter). A construct composed of the firefly luciferase gene under the control of the ovine β -lactoglobulin promoter was used to provide a rapid indication of milk protein promoter driven gene expression. Pools of stable transfectants were grown to confluency in 6 well dishes and induced with lactogenic hormones, prolactin (5 μ g/ml) and dexamethasone (1 μ M) or in the absence of lactogenic hormones (uninduced). Luciferase activity was assayed after 3 days. This data demonstrate that fetal fibroblasts are capable of

supporting expression of milk specific genes in response to lactogenic stimuli when transfected with the prolactin receptor and other components of the prolactin signalling pathway and that this is enhanced
5 by the presence of sodium butyrate.

Table 5 Milk specific reporter gene expression in modified fetal fibroblasts

10

		Luciferase units/mg protein			
		Expt 1		Expt2	
Transfection	Na	unind.	ind.	unind.	ind.
	butyrate				
No DNA	+	3	0	0	7
No DNA	-	0	0	0	0
BLUC	+	3	1	536	917
BLUC	-	0	0	108	28
BLUC+PRL	+	894	6360	220	610
BLUC+PRL	-	233	237	196	3373
BLUC+STAT5a	+	872	115935	17827	26104
BLUC+STAT5a	-	141	96	76	38
BLUC+PRL	+	7644	18543	367	10829
+STAT5a					
BLUC+PRL+	-	186	296	35	22
STAT5a					

Because the presence of individual components of the prolactin signal transduction pathway will be unknown in most cells to be used for nuclear transfer, a preferred
15 embodiment of this approach is the addition of all

factors.

B. Fusion of fibroblasts with mammary epithelial cells.

- 5 Milk protein gene expression can be induced in stably transfected fibroblasts by fusion with mammary epithelial cells which are functionally differentiated or capable of undergoing functional differentiation. In this way cytoplasmic and cell surface components
10 required for the activation of milk protein gene expression are made available to the fibroblast nuclei. The heterokaryon fusion products can be screened directly for expression of the transgene.
- 15 Cross-specific cell fusion.
- PDFF2 cells stably transfected with a construct comprising the green fluorescent protein (GFP) (Chalfie et al. op. cit., 1994) gene under the control of the β -lactoglobulin promoter were fused with a conditionally
20 transformed murine epithelial cell line KIM-2, which has previously been demonstrated as capable of expressing a broad range of milk proteins in vitro (Gordon K.E. et al. Intracellular Signaling in the mammary gland, Wilde C.J., Peaker M. and Knight CH.
25 Plenum, London. 1985) transfected PDFF2 cells and KIM-2 cells were fused by co-centrifugation in the presence of a fusogenic reagent e.g. polyethylene glycol 1000, 6000, or inactivated Sendai virus. Conditions for heterokaryon formation were optimised by assaying the
30 number of heterokaryons formed. Murine and ovine nuclei in heterokaryotic cells were identified by staining

with Hoechst 33258, which produces characteristic different patterns of nuclear staining in these species. Following induction with lactogenic hormones the expression of the transfected gene expression from
5 fibroblast nuclei was visualised as GFP fluorescence in heterokaryons

Con-specific cell fusion.

In some circumstances it may be preferable to induce
10 gene expression by fusion with cells of the same species.

Ovine mammary epithelial cells were prepared from mammary tissue by a method originally developed in
15 mouse by Barcellos-Hoff and Bissell (*Development* 105, 223-235, 1989). The excised glands from pregnant or lactating animals were minced into 1-2mm fragments and incubated with digestive enzymes such as collagenase and dispase to dissociate the tissue. This released
20 loosely associated fibroblasts and adipocytes. Differential centrifugation was used to enrich for the epithelial component. The cellular composition of the cultures was established on the following criteria: cell morphology, immunohistochemical detection of
25 epithelial cell-specific cytokeratin markers such as keratin 18 and 19 (Lane, E.B. *J. Cell Biol.* 92, 665-673, 1982 and Stasiak, P.C. et al. *J. Invest. Dermatol.* 5, 707-716, 1989) and functionally by expression of endogenous milk protein expression e.g. casein.

30

Ovine mammary epithelial cells were fused with PDFF

fetal fibroblasts stably transfected with a β -lactoglobulin driven GFP reporter construct. Application of lactogenic stimuli resulted in GFP expression in heterokaryons, indicating induction of milk gene expression.

These techniques were applied to gain predictive information regarding the choice of transfected cell clones for nuclear transfer as below.

10

PDFF cell clones transfected with a transgene construct were isolated, a sample of each was cryopreserved at an early stage, and a portion grown on for analysis for suitability for nuclear transfer as described in example 2. A further round of screening was then carried out to determine the level of transgene expression in vitro. Samples of each fibroblast clone were modified to allow induction of BLG directed transgene expression. The level of transgene expression in each clone was assayed by RNA and protein analysis and those clones which displayed the highest levels of expression were identified. The unmodified frozen sample corresponding to those clones was then thawed and cells used as a nuclear donors as described in example 2.

25

Example 4. Placement of transgenes at optimal sites

This example demonstrates how transgenes can be placed at genomic locations previously identified as supporting favourable transgene expression.

30

Cell mediated transgenesis is an ideal way to identify locations in the genome which are favourable to transgene expression. Far more individual integration sites can be screened in transfected cells than is
5 feasible in transgenic animals. Once a location shown to support high expression has been identified, other transgenes can be targeted to the same position.

A transgene construct was generated which was flanked by
10 recognition sites for a site specific recombinase (e.g. bacteriophage P1 Cre recombinase LoxP sites) and a recognition site for a rare cutting endonuclease (e.g. I-Sce I). The construct was transfected into cells, independent transfected clones were screened for
15 transgene expression as described in example 3 and a favourable clone used to generate transgenic animals by nuclear transfer as described in example 3. This provided confirmation of the ability of the cell clone to produce a healthy animal by nuclear transfer.

20

Other transgenes can then be targeted to the same site in cells in vitro using the scheme outlined in Figure 1.

25 Example 5. Sheep produced by nuclear transfer from embryo derived cells

This example demonstrates the production of sheep by nuclear transfer from another cell type, providing further evidence of the general applicability of the
30 present invention.

A Poll Dorset sheep embryo was flushed from the oviduct of a ewe at 9 days post coitum and placed in culture. The embryonic disc was isolated by microdissection and cultured in the presence of mitotically inactivated
5 feeder cells. After 8 days the explanted embryonic disc was disaggregated and cells replated onto a fresh feeder layer. After a further 7 days growth a colony of extended very flat cells became apparent, this was isolated and grown further in the absence of feeder
10 cells. The cells derived are termed SEC1 and do not resemble the TNT4 cells derived by a similar procedure by Campbell et al., (op. cit.) 1996. Frozen stocks of SEC1 cells were prepared at passage 4 (27 days in culture) and a passage 5 (30 days in culture). PCR
15 analysis using sex specific primers (Figure 3) showed SEC1 cells to be male.

Analysis of the suitability of SEC1 cells for nuclear transfer was carried out as in example 2 for fetal
20 cells. Counts of metaphase chromosomes of SEC1 at passage 8 revealed a modal number 54. Serum starvation of SEC1 cells induced reversible quiescence (Table 6).

Table 6

Reversible arrest of ovine embryo derived cell division
by serum starvation

		cell count (X10 ⁴)	
5	Days in 0.5%	in 0.5% serum	4 days after 10% serum restored
	0	2	
	2	2.1	7.1
10	3	1.9	6.5
	6	1.6	4.8

SEC1 cells were prepared for nuclear transfer by serum
starvation for 5 days and nuclei transfer carried out as
15 in example 2. Table 7 shows the results of the nuclear
transfer experiment.

Table 7 Nuclear transfer from embryo derived cells

20		Cell Type SEC1
	Number of reconstructed embryos	385
	Number of morulae + blastocysts	126
	Number of embryos transferred	87
25	Number of pregnancies	15
	Number of live lambs	4

The 4 lambs born are all Poll Dorset in appearance, male
(Figure 3) and identical to SEC1 cells by microsatellite
30 analysis using 4 primer pairs (Figure 2).

Example 6. Primary cultured cells may be modified to extend their lifespan

This example demonstrates how transgene analysis can be facilitated in short lived cells by conditional
5 immortalisation of a portion of each transfected clone.

Although unmodified SEC1 cells can produce lambs, SEC1 cell mediated transgenesis is hindered by the relatively short lifespan of the cells in culture. SEC1 cells grow
10 for approximately 12 passages (approximately 7 weeks) in culture, insufficient to derive and analyse stable transfectant clones by drug selection. As this may apply to other primary cells, we describe here how the problem is overcome.

15

Individual primary SEC1 cells were microinjected with the transgene construct and plated such that individual colonies arose from each microinjected cell. Once sufficient time had elapsed to allow degradation of non-
20 integrated DNA (approximately 7 days), samples of a few cells were taken from each clone and analysed for the presence of the transgene by polymerase chain reaction.

Clones containing the transgene were then split, one portion cryopreserved and one portion transfected with a
25 construct expressing an immortalising gene. In this example we have used a construct PGKSV40Tts (Figure 4) which expresses a temperature sensitive SV40 T antigen (Jat P. et al., *Proc. Natl. Acad. Sci. USA* 88, 5096-5100, 1991) under the control of the human phosphoglycerate
30 kinase gene promoter. The PGKSV40Tts gene cassette is flanked by *LoxP* sites to allow removal if necessary. 5

51

stably transfected SEC1 clones were grown for an extended period and in each case the lifespan (18-19 passages, ~3 months) extended beyond the natural lifespan of primary SEC1 cells.

5

Immortalised clones can then be expanded and analysed at will. Where necessary, analysis of transgene expression in the absence of T antigen expression can be achieved by growth at the non-permissive temperature (39°C). As
10 in examples 3 and 4, once a suitable clone was identified, the cryopreserved sample of non-transformed cells could be thawed and used for nuclear transfer.

15

CLAIMS

1. A process for producing a nuclear donor cell, the process comprising:
 - 5 transfecting a cell with a transgene; and
 - screening the cell for a desired phenotype.
2. A process as claimed in claim 1, wherein the process further includes assessing the suitability of
10 the cell for nuclear transfer.
3. A process as claimed in claim 1 or claim 2, wherein the screening for a desired phenotype is *in vitro*.
- 15 4. A process as claimed in any one of claims 1 to 3, wherein the screening for a desired phenotype is by analysis of transgene expression characteristics.
5. A process as claimed in any one of claims 1 to 4,
20 wherein the cell is an adult somatic cell, an embryonic somatic cell or a foetal somatic cell.
6. A process as claimed in any one of claims 1 to 5, wherein the transfected cell models a tissue of
25 interest.
7. A process as claimed in any one of claims 1 to 6 which further includes the step of preparing the cell for nuclear transfer.

8. A process as claimed in any one of claims 1 to 7, further including the step of transgene placement at a favourable site.

5 9. A nuclear donor cell produced by a process as claimed in any one of claims 1 to 8.

10. A process for obtaining predictive information of the phenotype of a non-human transgenic animal, the
10 process comprising:

transfecting a cell with a transgene; and
screening the cell to obtain phenotypic information.

15 11. A process for reconstituting a non-human animal embryo, the process comprising:

transfecting a cell with a transgene;
analysing the phenotype of the cell;
inducing the cell into state suitable for nuclear
20 transfer; and
transferring the nucleus of the cell into a suitable recipient cell.

12. A process as claimed in claim 11, wherein the
25 analysis of the phenotype of the cell is by analysis of transgene expression characteristics.

13. A process as claimed in claim 11 or claim 12, wherein the cell is an adult somatic cell, an embryonic
30 somatic cell or a foetal somatic cell, preferably a mammary epithelial cell, a fibroblast cell, an

endothelial cell or a sub-endothelial cell.

14. A process as claimed in any one of claims 11 to 13, wherein the animal is an ungulate species.

5

15. A process as claimed in claim 14, wherein the animal is a cow, bull, pig, goat, sheep, camel or water buffalo.

10 16. A process as claimed in any one of claims 11 to 15, wherein the suitable recipient cell is an oocyte.

17. A process as claimed in claim 16, wherein the oocyte is enucleate.

15

18. A process as claimed in any one of claims 11 to 17, wherein the transfected cell models a tissue of interest.

20 19. A process as claimed in any one of claims 11 to 18, further including the step of transgene placement at a favourable site.

20. A reconstituted non-human animal embryo produced by
25 a process as claimed in any one of claims 11 to 19.

21. A non-human animal produced from a reconstituted animal embryo as claimed in claim 20.

30 22. A process for producing an animal, the process comprising:

(a) reconstituting an animal embryo as claimed in any one of claims 11 to 19;

(b) causing an animal to develop to term from the embryo; and

5 (c) optionally, breeding from the animal.

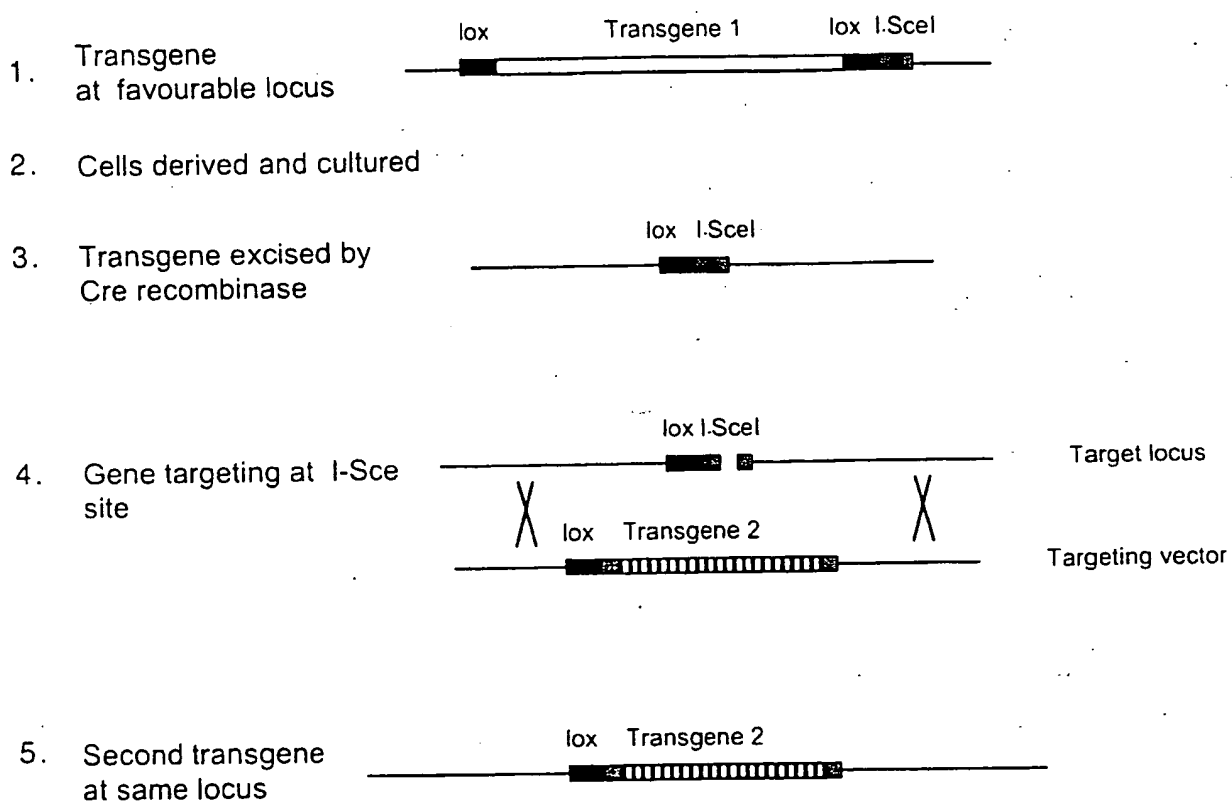
23. An animal produced by the process of claim 22.

24. A process for targeting a transgene to a location
10 of interest, the process comprising: selecting a nuclear donor cell produced by the process of claim 8 or a reconstituted animal embryo produced by the process of claim 19 or an animal developed from an embryo produced by the process of claim 19 and using a gene targeting
15 vector to place the target transgene at the target locus.

25. A nuclear donor cell, reconstituted animal embryo or animal produced by the process of claim 24.

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FIGURE 1.
TRANSGENE PLACEMENT AT A LOCUS MARKED BY
A RARE ENDONUCLEASE RECOGNITION SITE



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FIGURE 2.
MICROSATELLITE ANALYSIS OF NUCLEAR
TRANSFER CELLS AND LAMBS

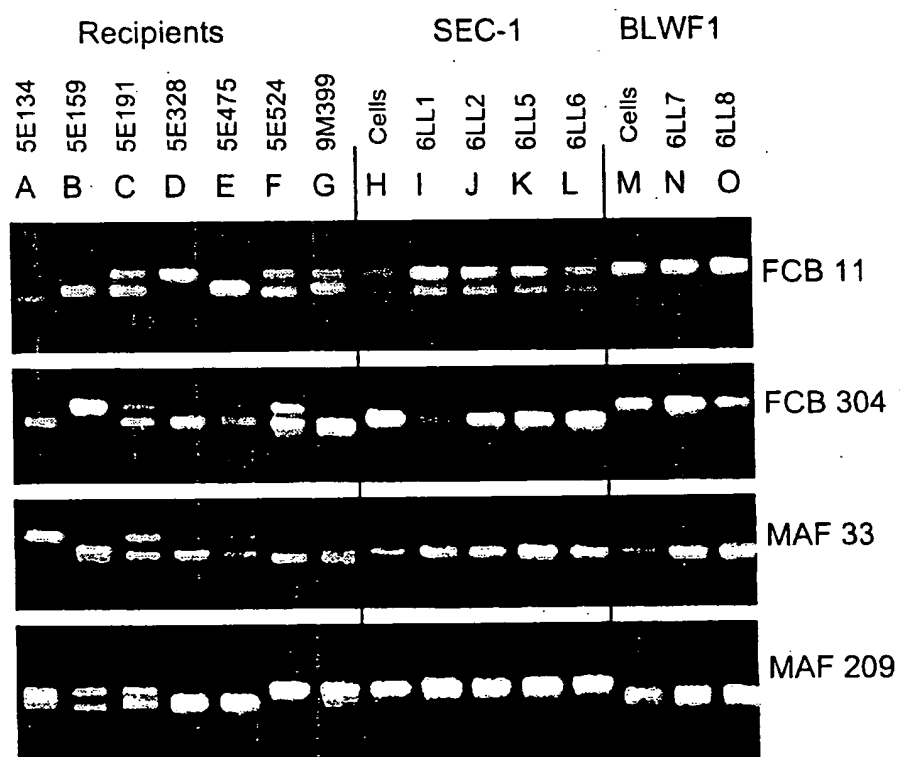


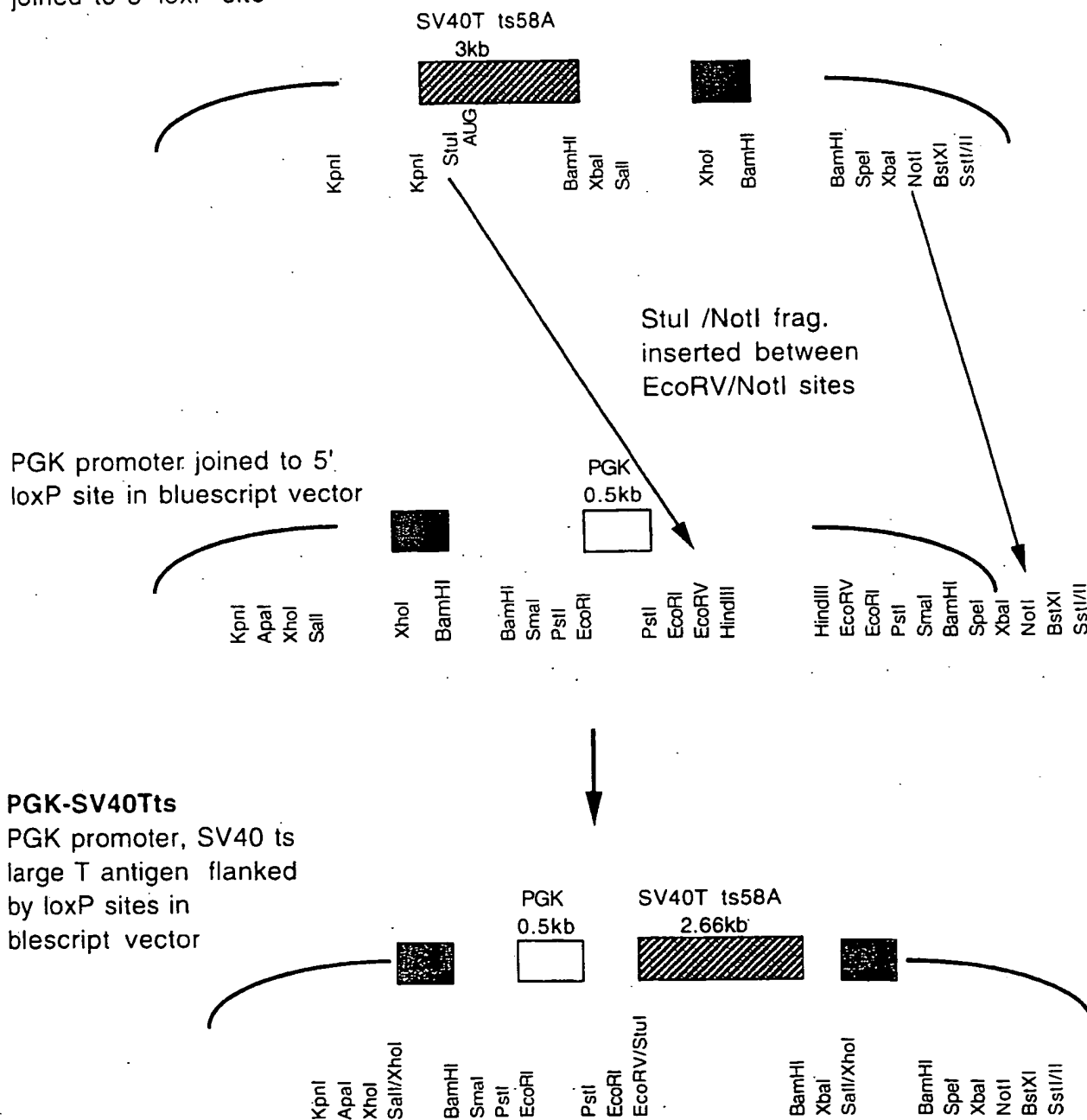
FIGURE 3.
SEX ANALYSIS OF NUCLEAR
TRANSFER CELLS AND LAMBS



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FIGURE 4.
CONSTRUCTION OF PGKSV40Tts

SV40Tts 58A fragment
joined to 3' loxP site



A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/00 A01K67/027 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CIBELLI, J.B. ET AL.: "Production of germline chimeric bovine fetuses from transgenic embryonic stem cells" THERIOGENOLOGY, vol. 47, no. 1, 17 December 1996 - January 1997, page 241 XP002067034	1,3-5,9, 10,12-15
Y	see the whole document & Annual conference of the international embryo transfer society, Nice, France, January 12-14 1997	1-7,9, 11-17, 20-23
	---	-/--

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

9 June 1998

Date of mailing of the international search report

03.07.98

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 20042 A (PPL THERAPEUTICS SCOTLAND LTD ;MCWHIR JAMES (GB); STACEY ALEXANDER) 27 July 1995	1,3-5,9,10
Y	see the whole document	1-7,9,11-17,19-23
X	--- MCWHIR, J. ET AL.: "Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a non-permissive genetic background" NATURE GENETICS, vol. 14, no. 2, 1996, pages 223-226, XP002067434 see the whole document	1,3-5
Y	--- WO 96 07732 A (ROSLIN INST EDINBURGH ;MCWHIR JAMES (GB); CAMPBELL KEITH HENRY ST0) 14 March 1996 see the whole document	1-7,9,11-17,20-23
T	--- EP 0 774 510 A (MEIJI MILK PROD CO LTD) 21 May 1997 see the whole document	1
X	& WO 95 34636 A 21 December 1995	
X	--- US 5 487 992 A (CAPECCHI MARIO R ET AL) 30 January 1996	1,3-6,8-10,24,25
Y	see the whole document	2,7,11-13,16-23
X	--- EP 0 633 314 A (TAKEDA CHEMICAL INDUSTRIES LTD) 11 January 1995	1,3-6,8-10,24,25
Y	see the whole document	2,7,11-13,16-23
X	--- WO 91 01140 A (CELL GENESYS INC) 7 February 1991	1,3-6,8-10,24,25
Y	see the whole document	2,7,11-13,16-23
	--- -/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHNIEKE, A.E. ET AL.: "Human Factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts" SCIENCE., vol. 278, no. 5346, 19 December 1997, LANCASTER, PA US, pages 2130-2133, XP002067036 see the whole document	1-7, 9-18, 20-23
P,X	STICE, S.L. ET AL.: "Cloning: new breakthroughs leading to commercial opportunities" THERIOGENOLOGY, vol. 49, no. 1, January 1998, pages 129-138, XP002067435 see page 133, paragraph 4 - page 135, paragraph 3	1-25
P,X	WO 97 37009 A (UNIV MASSACHUSETTS A PUBLIC IN) 9 October 1997 see claims 30-41; examples 2,3	1
P,X	WO 97 49804 A (BAYLOR COLLEGE MEDICINE) 31 December 1997 see the whole document	1,24,25
X	FIERING S ET AL: "AN "IN-OUT" STRATEGY USING GENE TARGETING AND FLP RECOMBINASE FOR THE FUNCTIONAL DISSECTION OF COMPLEX DNA REGULATORY ELEMENTS: ANALYSIS OF THE BETA-GLOBIN LOCUS CONTROL REGION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 18, 15 September 1993, pages 8469-8473, XP000563921 see the whole document	1,3,5, 8-10,24, 25
P,A	WO 97 07669 A (ROSLIN INST EDINBURGH ;CAMPBELL KEITH HENRY STOCKMAN (GB); WILMUT) 6 March 1997 cited in the application see the whole document	5,13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00544

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 11-19 and 22 might be directed to a method of surgery practised of the animal body, the search has been carried out completely
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 98/00544

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9520042	A	27-07-1995	AU 1461895 A EP 0741781 A JP 9508020 T	08-08-1995 13-11-1996 19-08-1997
WO 9607732	A	14-03-1996	AU 3395695 A	27-03-1996
EP 0774510	A	21-05-1997	AU 2682895 A CA 2192982 A WO 9534636 A	05-01-1996 21-12-1995 21-12-1995
US 5487992	A	30-01-1996	US 5464764 A US 5627059 A US 5631153 A	07-11-1995 06-05-1997 20-05-1997
EP 0633314	A	11-01-1995	JP 7246040 A CA 2127412 A US 5625123 A	26-09-1995 07-01-1995 29-04-1997
WO 9101140	A	07-02-1991	AU 633958 B AU 6150590 A CA 2037907 A EP 0437576 A JP 4501510 T US 5574205 A US 5416260 A US 5413923 A	11-02-1993 22-02-1991 26-01-1991 24-07-1991 19-03-1992 12-11-1996 16-05-1995 09-05-1995
WO 9737009	A	09-10-1997	AU 2219497 A	22-10-1997
WO 9749804	A	31-12-1997	AU 3507297 A	14-01-1998
WO 9707669	A	06-03-1997	AU 6831096 A GB 2318578 A	19-03-1997 29-04-1998